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Correlation of Histocompatibility Reactions with Fusion Between Conspecifics in the Solitary Urochordate *Styela plicata*

ELIZABETH A. KINGSLEY[1], DAVID A. BRISCOE, AND DAVID A. RAFTOS[2]

School of Biological Sciences, Macquarie University, New South Wales 2109, Australia

Abstract. Previous transplantation analysis has identified a sensitive histocompatibility system in the solitary urochordate *Styela plicata* that obeys genetic transplantation "rules" identical to those of vertebrates. The current study demonstrates that histocompatibility acts to prevent fusion between conspecifics in sedentary aggregations of this species. Pairs of naturally fused individuals were present at low frequencies (0.06%) in natural populations. Comparative transplantation analysis, in which grafts were transferred from fused donors to panels of single (non-paired) recipients, confirmed that such fusion occurs only between individuals of identical histocompatibility tissue type. Ninety-four percent of recipients yielded identical responses to allografts from both members of fused pairs, suggesting that fused individuals expressed concordant histocompatibility tissue types. In contrast, only 69% of hosts receiving allografts from pairs of randomly selected unfused individuals yielded identical responses to both donor tissues. Allozyme analysis confirmed the specificity of this correlation between fusion and shared histocompatibility type. Fused individuals retained distinct genetic identities for electrophoretically resolved loci independent of tissue type.

In light of these results, it is argued that the role of histocompatibility systems in preventing fusion may have been a strong selective force in the evolution of allogeneic recognition.

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[1] Present address: Clinical Immunology Research Centre, Sydney University, N.S.W. 2006, Australia.

[2] Present address: Department of Anatomy, UCLA School of Medicine, University of California, Los Angeles, California 90024.

Introduction

Invertebrate histocompatibility responses have frequently been implicated in the prevention of fusion between conspecifics (Bigger *et al.*, 1982; Buss *et al.*, 1984; Smith and Hildemann, 1984; Buss and Green, 1985; Kaye and Reiswig 1985; Van de Vyver *et al.*, 1985; Mukai and Shimoda, 1986). In colonial urochordates, allogeneic discrimination prevents fusion between incompatible colonies, but allows the integration of asexually derived zooids and other compatible individuals (Tanaka, 1973; Mukai and Watanabe, 1974; Katow and Watanabe, 1980; Taneda and Watanabe, 1982a, b). Despite this demonstrable regulatory function in recruitment among colonial species, the physiological role of histocompatibility in solitary urochordates has not been investigated.

However, recent evidence suggests that solitary species possess both the capacity for fusion *in situ* and sensitive histocompatibility mechanisms. Schmidt (1982) has shown that aggregative settlement in the solitary urochordate *Molgula complanata* results in the fusion of tunic matrices among 20% of metamorphosed adults. This finding complements tissue transplantation studies in *S. plicata*. In this species, approximately 75% of interpopulation tunic allografts undergo chronic rejection which subserves genetic transplantation "rules" identical to those of vertebrates (Raftos *et al.*, 1987a, 1988).

The aim of the current study is to correlate such observations by identifying a specific requirement for shared histocompatibility tissue type among individuals of *S. plicata* that have undergone fusion *in situ*. In doing so, the significance of conspecific fusion as a potential selective stimulus for allorecognition will be extended beyond that of specific adaptation to colonial life histories.

Materials and Methods

Animals

Fifty-two pairs of putatively fused *S. plicata* specimens were collected from shark netting or pontoons at two sites on Sydney Harbor, Balmoral Beach, and Birkenhead Point, Australia. These sites were separated by 8 km of estuarial coastline. Both areas maintained large, stable populations of *S. plicata*. Putatively fused pairs were initially identified *in situ* as individuals living in close association such that large areas of their tunics were in direct contact. The frequency of these pairs, relative to single (unpaired *in situ*) animals at the Birkenhead Point site, was determined as a function of mean population density based on counts of all individuals in 10 randomly selected quadrats. An additional 384 single Balmoral Beach individuals were collected for use as controls or graft recipients.

During the carmine retention studies and prior to grafting, specimens were held in 30-l polyethylene aquaria under constant aeration and filtration. After grafting, recipients were cultured *in situ*. They were enclosed in plastic mesh cages secured to the 0.1 m tide level on shark netting at Balmoral Beach.

Morphological analysis of fusion

The identification of individuals exhibiting fusion was confirmed by removing 1 cm^3 blocks of tunic tissue incorporating the interface between putatively fused animals. For comparison, similar blocks were removed from the epidermal layer of 20 single (unpaired *in situ*) individuals. Tissue blocks were fixed for 24 h in formalin (10% v/v in filtered seawater) and then sectioned (10 μm) with a freezing microtome (Cryostat, Massachusetts). Sections were stained with haemotoxylin and eosin (Humason, 1972) prior to bright field microscopical inspection. Based on this examination, "fused" pairs were defined as those lacking part or all of an eosinophilic layer at their point of contact (Fig. 1). Closely associated individuals which retained a continuous eosinophilic boundary at the interface were defined as "non-fused" pairs (Fig. 1).

Further resolution of tunic fusion was provided by scanning electron microscopy (SEM). The blocks of tunic tissue remaining after sectioning for bright field analysis were washed by immersion in filtered seawater for 24 h before being critical point dried using liquid CO_2 as the transition medium. Dried tissue was mounted on aluminum stubs with the microtome cut surface facing upwards and then sputter coated with gold (4 min, 20 mA). Specimens were inspected with a Joel 840 scanning electron microscope at an accelerating voltage of 10 kV. (Joel Australasia Ltd., Sydney).

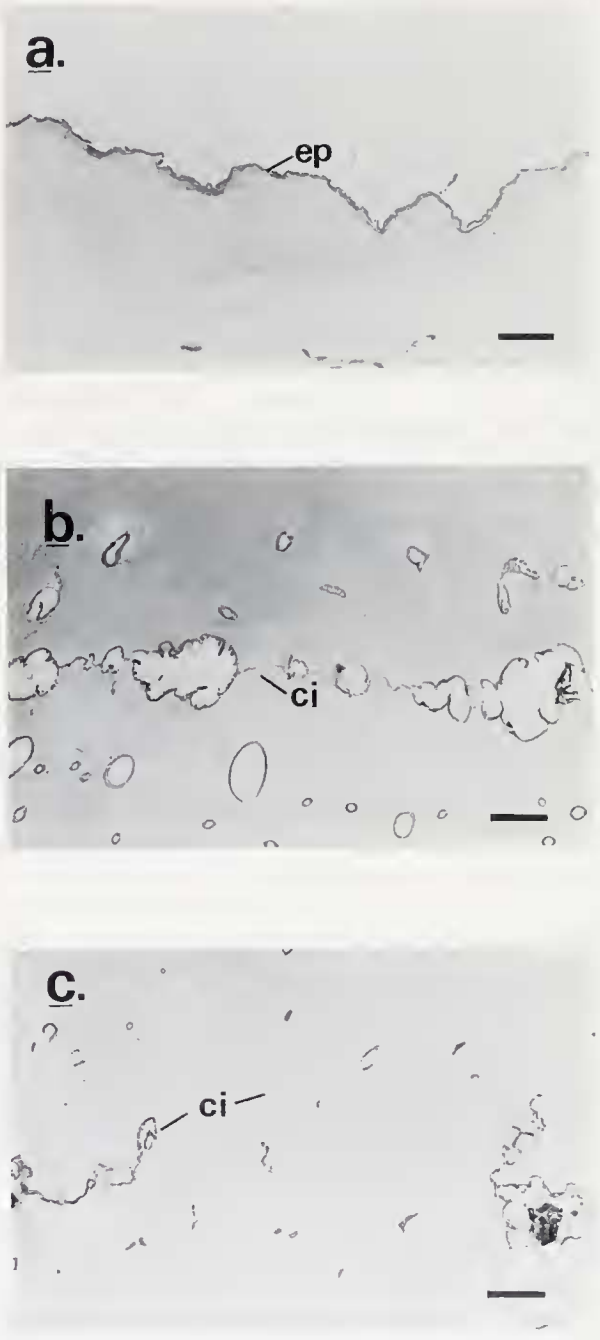

Figure 1. Bright field micrographs of the: (a) outer epidermis of a single (unpaired) individual. (b) contact interface between two "non-fused" animals. (c) contact interface between "fused" individuals. ci = contact interface, ep = epidermis. Scale bars = 1 mm.

Analysis of vascular connection

The degree of vascular connection between closely associated individuals was initially investigated by bright field microscopical analysis of frozen sections. The fre-

quency of tunic vessels was determined within 5 mm of the contact interface between putatively fused animals and within 5 mm of the epidermis of single (unpaired *in situ*) animals.

Vascular interaction was further delineated by the transmission of particulate carmine between fused individuals. One animal from each of four fused and four non-fused pairs (as defined above) was injected with 400 μl carmine (10% w/v in filtered seawater). Carmine solutions were injected into blood sinuses of the mantle lying directly beneath the tunic. Injected animals were subsequently maintained in aquaria. To identify any passive uptake of excreted carmine, single control animals were placed with the injected pairs in each aquarium.

Three days after injection, 200 μl samples of hemolymph were removed from scalpel wounds made in the right posterior tunic and mantle of both animals from each pair and control individuals. The frequency of carmine particles in these samples was determined using a hemocytometer.

Comparative histocompatibility tissue typing

The relationship between fusion and histocompatibility was assessed by comparative tissue typing based on established tunic transplantation techniques (Raftos *et al.*, 1987a, b, 1988) and previously defined genetic "rules" for transplantation rejection in *S. plicata* (Raftos *et al.*, 1988; Raftos and Briscoe, 1989). Transplants, in the form of cylindrical cores, were removed from the tunic of donor individuals by obliquely inserting a length of 3 mm diameter stainless steel syringe tubing. Grafts were implanted into holes in the tunic of the host that had been prepared by similar insertion of syringe tubing and removal of detached cores. This procedure was used to provide two forms of graft. Autografts were transferred within a single individual so that they assumed a position 1 cm anterior to their original location on the dorsal stolonic margin. In contrast, allografts represented tissue orthoptically transferred between separate individuals.

The relative tissue types of fused individuals were determined by transplanting allografts from nine fused pairs to panels of randomly chosen single Balmoral Beach individuals. Each animal in the recipient panels received two simultaneous allografts, one from each member of a particular fused pair. The two allografts were separated from each other by at least 1 cm. The size of the recipient panel derived from each fused pair was dependent upon the number of grafts that could be extracted from the donors. Fused pairs usually provided sufficient grafts for twenty recipients.

This tissue typing procedure was repeated using single animals as donors to provide estimates of tissue type concordance in the general (unfused) population. In these trials, 20 single Balmoral Beach individuals were randomly allocated into 10 donor pairs, providing allografts for panels of approximately 10 recipients per pair.

Estimates of the frequency for graft loss arising from technical failure of the transplantation procedure were obtained by implanting dual autografts into a further 60 single Balmoral Beach animals.

The viability of grafts was determined 65 days after grafting. Assessment at this time provided an accurate distinction between compatible and incompatible grafts (Raftos *et al.*, 1987a). Mortality among grafted animals was such that graft viability could be assessed in 81, 68, and 43 individuals that had received tissue from fused pairs, paired single animals, or autogeneic combinations, respectively. Tissue incompatibility was defined as the complete rejection of the graft from the graft bed, whereas compatibility was characterized as the retention of some or all of the graft. Recipients were deemed to have yielded a concordant response to transplantation if both grafts from separate donors were retained (++) or rejected (−−). Non-concordant responses occurred when a graft from one donor was retained, while that from the other donor was rejected (+−).

Electrophoretic analysis of genetic variation

The similarity of fused individuals for factors other than histocompatibility was determined electrophoretically. Gonads and pharyngeal baskets were removed from both animals in nine fused pairs, and from 18 single Balmoral Beach animals that had been randomly allocated into pairs. Tissue samples were homogenized in 0.1 *M* phosphate buffer and subjected to starch gel electrophoresis (Harris and Hopkinson, 1976). Gels were stained for the enzymes: phosphoglucose isomerase, E.C. 5.3.1.9. (PGI); aspartate aminotransferase, E.C. 2.6.1.1. (AAT); and phosphoglycerate kinase, E.C. 2.7.2.7. (PGK-2), by the methods of Richardson *et al.* (1986).

Results

Morphological analysis of fusion

Based on histological analysis of the contact interface, the 52 putatively fused pairs identified *in situ* fell into two distinct groups. Eighty-three percent of putatively fused animals failed to exhibit fusion of their tunic matrices, and thus were considered to be closely associated but "non-fused" pairs. Bright field inspection of frozen sections revealed an eosinophilic layer at the contact interface in these non-fused pairs (Fig. 1b). SEM resolved this layer as a dense tunic boundary (Fig. 2b) similar to the proteinaceous (Goodbody, 1974) epidermal layer of sin-

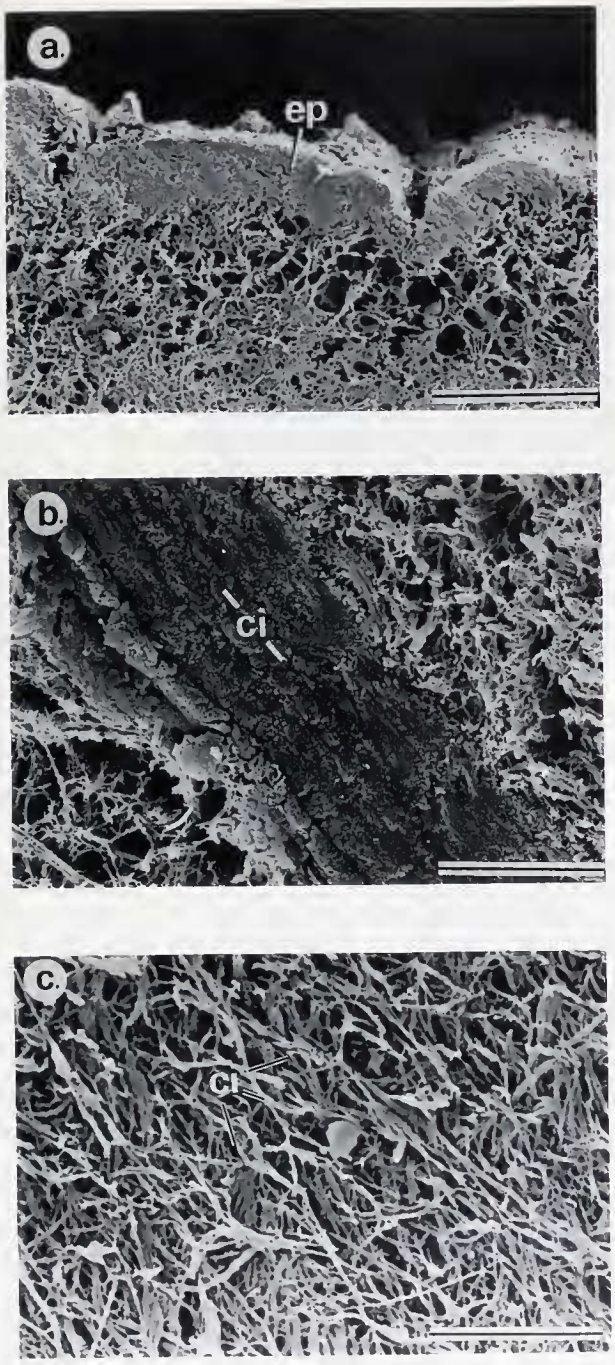

Figure 2. SEM micrographs of: (a) the epidermis of a single animal. (b) Dense tunicin boundaries at the contact interface between individuals in a "non-fused" pair. (c) Interdigitation of tunicin fibres at the contact interface between "fused" animals. ci = contact interface; ep = epidermis. Scale bars = 10 μm.

gle animals (Figs. 1a, 2a). The boundary prevented the interdigitation of extracellular tunic fibers between individuals, allowing animals in non-fused pairs to be pried apart with little effort.

The remaining 17% of putatively fused individuals identified *in situ* exhibited an obvious fusion of their tunic matrices. In these "fused" pairs, eosinophilic boundaries were either intermittent or absent from the point of contact (Fig. 1c). This absence of dense boundaries between fused animals was reflected in SEM by the interdigitation of tunicin fibers between individuals (Fig. 2c) yielding a confluent tunic matrix.

Fusion was most often associated with a unique external morphology (Fig. 3). Contact was developed, between individuals of approximately equal size, along the ventral stolonic margin, so that the atrial siphons were not adjacent. Such fused animals could not be pried apart without causing extensive damage to the tunic. Based on the mean population density at the Birkenhead Point collection site, the relative frequency of fused pairs conforming to these characteristics was 6.4×10^{-4}.

Vascular connection of fused animals

The distinction between fused and non-fused pairs was further delineated by the penetration of vascular elements into the contact zone between individuals. Figure 4 reveals that non-fused pairs exhibited a similar pattern of vascular penetration to that of the epidermis in single (unpaired *in situ*) animals. The maximum frequency of tunic vessels occurred 3–5 mm from the contact zone in non-fused pairs and fell to 0 at the interface, indicating a lack of vascular connection between animals. In contrast, pairs exhibiting fusion of tunic matrices showed peak tunic vessel frequencies closer to the point of contact (1–2 mm) and retained substantial vascular activity at the interface, confirming vascular connection between fused animals.

The fate of injected carmine particles indicated that this vascular connection of fused pairs allowed substantial interchange of hemolymph components. In fused pairs, 21 $\pm$ 2% of the carmine particles that had entered the hemolymph of injected animals were transferred to the second, uninjected individual. In contrast, uninjected animals in non-fused pairs accumulated only 0.8 $\pm$ 0.4% of the carmine found in the hemolymph of their partners. This did not differ significantly ($t_6 = 0.91$; $P > 0.05$) from the frequency of carmine in the hemolymph of uninjected single control animals that were cultured in the same aquaria as injected individuals. In these control individuals, minimal uptake of carmine occurred either through phagocytic ingestion of excreted carmine or via penetration of the atrium during bleeding.

Comparative histocompatibility tissue typing

The relationship between histocompatibility tissue type and fusion is shown in Table 1. If, as postulated,

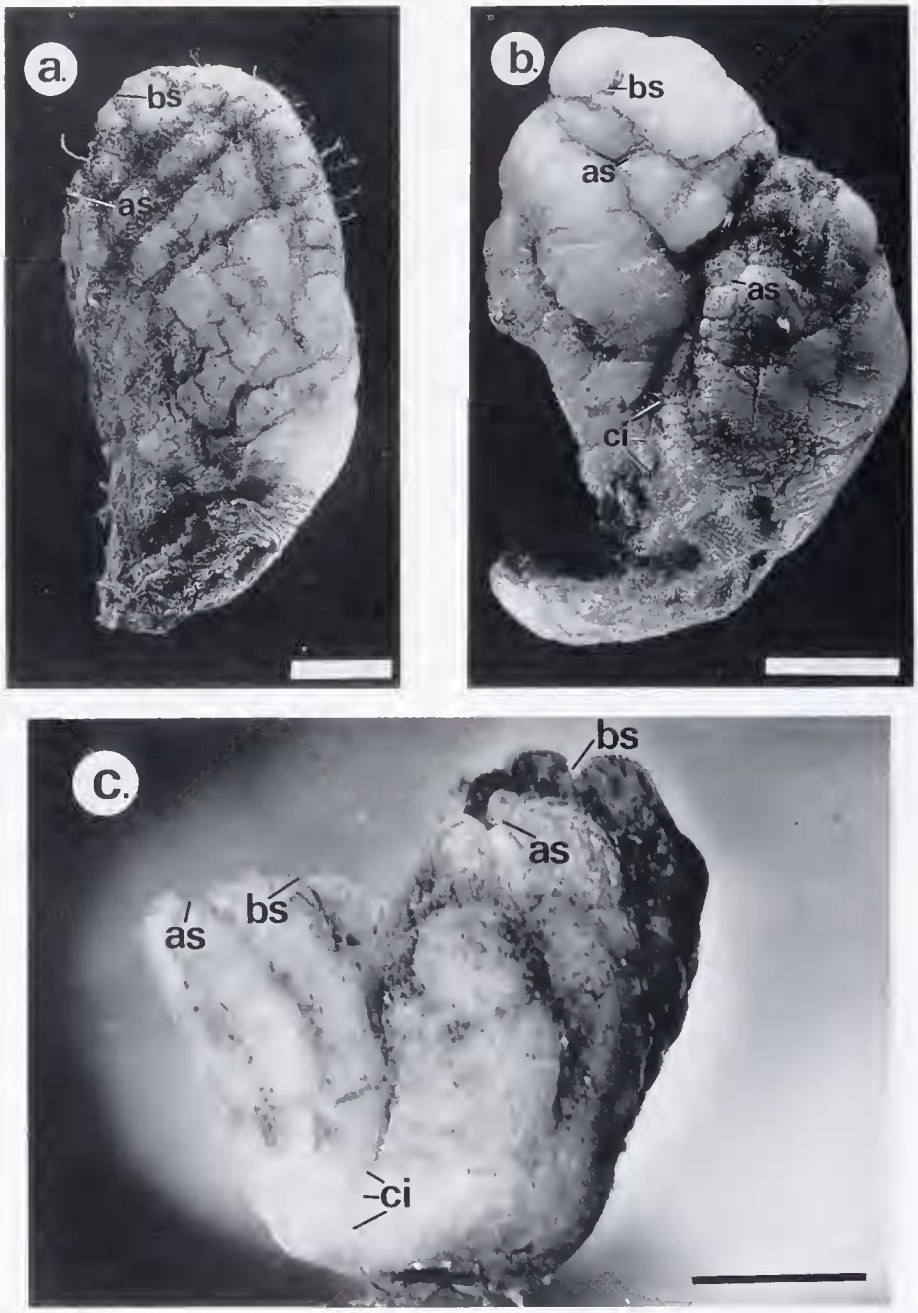

Figure 3. Typical external morphologies of: (a) single (unpaired) individuals. (b) "Non-fused" paired animals. (c) "Fused" individuals. as = atrial siphon, bs = buccal siphon, ci = contact interface between individuals. Scale bars = 1 cm.

fused individuals shared identical tissue types, it would be expected that recipients should yield identical, or concordant, responses to grafts from both members of a particular fused pair. Such expectations are based on previous genetic analysis which has shown that *S. plicata* obeys transplantation "rules" identical to those of vertebrates (Raftos *et al.*, 1988). Recognition of a single disparate histocompatibility haplotype between individuals will initiate graft rejection. Hence, to fuse, individuals should be of identical genotype with respect to histocompatibility and so should invoke concordant responses among panels of naive recipients in tissue typing trials. The data presented in Table I suggests that this is, indeed, the case. Ninety-four percent of individuals receiv-

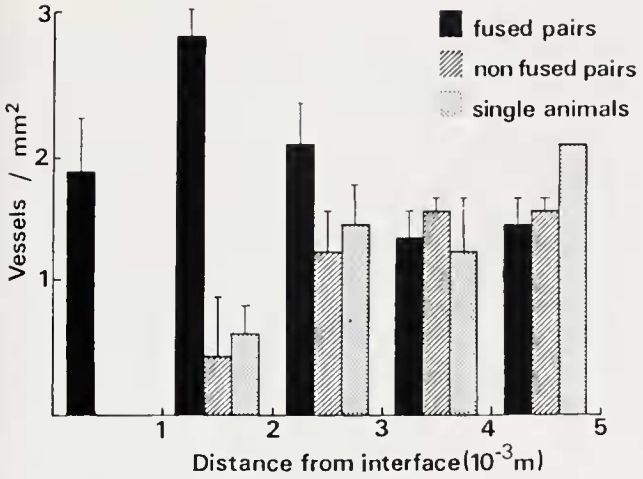

Figure 4. Mean frequencies of blood vessels at various distances from the contact interface between "fused" individuals or "non-fused" animals, or from the epidermis of single (unpaired) animals. Bars represent standard error (n ≥ 7).

ing allografts from fused pairs exhibited concordant transplantation responses. This was characterized by simultaneous retention (++) or rejection (−−) of grafts from both members of a donor pair. There was no significant difference ($\chi^2 = 1.06$, $P > 0.05$) between the frequency of non-concordant (+−) results among animals returning +− or ++ responses toward fused pairs (27.8%) and that observed for autografts (16.2%). Taking this favorable comparison with non-specific autograft loss into account, concordance toward fused pairs approached 100%, indicating an identity of donor tissue types.

In contrast, 30.8% of grafts from paired single animals returned non-concordant (+−) responses in recipient panels. This was significantly greater than the non-concordance frequency from fused pairs ($\chi^2 = 15.67$; $P < 0.05$). However, it corresponded ($\chi^2 = 2.98$; $P > 0.05$) with expected levels for a random binomial association of rejection and compatibility calculated from the total frequency for rejection (q; 0.71), such that randomly occurring non-concordance represented $\{2q(1-q)\} \times 100 = 41.2\%$.

Electrophoretic analysis of genetic variation

The similarity of electrophoretic genotypes between fused and single individuals is summarized in Table II. Electrophoretic analysis of three independent enzyme loci failed to reveal an overall genetic similarity between fused animals for factors other than histocompatibility that may have arisen through physiological chimerism or sibship. In 23% of fused pairs, individuals shared no

Table I

Frequency of concordant (++ or −−) and non-concordant (+−) transplantation reactions in individuals of Styela plicata *that had received either two allografts, one from each member of a fused pair, or two allografts from separate randomly paired single animals, or two autografts*

	Frequency (%)				
	Graft status[α]				
Donor animals	++	+−	−−	Total[β] rejection	Total concordance
Fused pairs (n[δ] = 81)	16.0	6.1	77.7	80.8	93.8
Paired single animals (n = 68)	13.2	30.8	55.8	71.3	69.1
Autografts (n = 43)	83.7	16.2	0.0	8.1	83.7

[α] ++, Denotes both grafts from separate donors accepted; +−, graft from only one donor intact; −−, grafts from both donors rejected.
[β] Total number of grafts rejected divided by the total number implanted.
[δ] Number of graft recipients scored 65 days after grafting.

allele at a single locus, while 41% shared one allele. Only 36% of fused individuals were genotypically identical at a given locus, and none shared identity at all three loci simultaneously. This pattern for fused pairs did not differ significantly ($\chi^2 = 1.06$; $P > 0.05$) from the level of genotypic similarity observed between pairs of randomly chosen single animals.

Discussion

The data presented here suggest that fusion will occur between conspecifics of *S. plicata* only when the individuals share common histocompatibility tissue types. Fu-

Table II

Similarity of electrophoretic genotypes for individuals in fused pairs and randomly selected pairs of single (unfused) animals

	Frequency (%)[α]		
Type of pair	No common allele	One common allele	Identical genotype
Fused pairs (n[β] = 9)	23	41	36
Single animals (n = 9)	23	27	50

[α] Data represent totals of independently determined frequencies for three different enzyme loci (PGI, AAT and PGK).
[β] Number of pairs tested.

sion in the natural environment was found to be a rare event involving a physiological confluence of fused animals. The eosinophilic epidermal layer observed in single animals, and between non-fused pairs, was absent from the contact interface between individuals in fused pairs. This allowed a vascular connection between fused individuals, exemplified by the penetration of tunic vessels across the contact interface. The exchange of particulate carmine between fused animals confirmed that this vascular penetration represented a functional connection.

It is postulated that such fusion is permitted by the sharing of histocompatibility tissue types between individuals. According to this hypothesis, the frequency of concordant response by recipient animals to grafts implanted from both members of a fused pair should approach 100%, reflecting identity of donor tissue types. Cases of non-concordant response should not occur if fused donors were of identical tissue type. Comparative histocompatibility tissue typing in this study has fulfilled these constraints. Concordant responses were recorded from 94% of animals receiving grafts from fused pairs. Taking into account the level of technical graft loss reflected by control autografts, this result approaches the predicted 100% frequency of concordance. In contrast, 31% of the individuals receiving grafts from arbitrarily paired single animals returned non-concordant responses consistent with a random association of rejection and compatibility. Clearly, grafts from single (unpaired *in situ*) animals exhibited independent histocompatibility tissue types, yielding a high frequency of non-concordant responses, while those from fused pairs shared histocompatibility types, and thus were subject to identical reactions.

The demonstrated correlation between tissue type and fusion may be explained by three alternative hypotheses. First, histocompatibility mechanisms may act to specifically prevent fusion. In contrast, fused individuals may share tissue types as a result of genetic chimerism arising from fusion. Finally, fusion may preferentially occur between animals of general genetic similarity (*i.e.*, sibs) and thus would reflect greater levels of tissue compatibility than the normal population. The electrophoretic analysis undertaken in this study supports the former option, that histocompatibility mechanisms specifically prevent fusion. Fused animals retained distinct genetic identities for characteristics other than histocompatibility tissue type. Their level of genetic similarity at three enzyme loci did not differ significantly from that of paired single animals. Thus, the observed correlation between histocompatibility and fusion could not have arisen from a broad genetic similarity derived from the development of genetic chimerism through physiological connection and is also unlikely to reflect sibship among fused animals. However, these results do not preclude a strong linkage disequilibrium between gene regions controlling histocompatibility and some other factor responsible for fusion.

The requirement for similarity of tissue type in fusion confirms that at least one function of the histocompatibility system in this sessile aggregative species is the prevention of fusion between conspecifics. Clearly though, histocompatibility is not the only factor preventing fusion. Previous genetic analysis has indicated that 9.5% of individuals in natural populations of *S. plicata* share histocompatibility tissue types, and so have the potential for fusion (Raftos and Briscoe, 1989). A far lower frequency of fused pairs was identified in the current study (0.06%). When taken with the consistent morphology of fused pairs, this suggests that other factors, such as the proximity, timing, and orientation of larval settlement, may play a substantial role in fusion. However, the paucity of fused pairs may also attest to the deleterious nature of fusion, reflecting a selective stimulus for the development of histocompatibility. It has been suggested that such selection pressure in invertebrates arises from germ or somatic cell parasitism, by which the individual genetic identity of fused animals may be eliminated (Buss and Green, 1985; Buss, 1982; Rinkevich and Weissman, 1987). However, electrophoretic analysis in this study has failed to identify a loss of genetic integrity in either gonadal or somatic tissue, even though substantial vascular connection was observed between fused animals. An alternative explanation is that fusion greatly increases the likelihood of infectious disease through the transfer of pathogens between individuals.

Regardless of its precise selective basis, the experimental correlation between fusion and histocompatibility in *S. plicata* remains clear. It suggests that the physiological functions of histocompatibility in invertebrates extend beyond the regulation of life histories in colonial organisms, and so may represent a generalized selective factor in the development of specific immunological recognition.

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